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**Foot pad dermatitis in growing turkeys is associated with
cytokine and cellular changes indicative of an inflammatory
immune response**

Mayne RK, Powell F, Else RW, Kaiser P, Hocking PM

Abstract

Cell and cytokine responses during the development of foot pad dermatitis (FPD) in growing turkeys were studied in a model system. The objective was to evaluate the hypothesis that FPD is an allergic response to the environmental materials. Hybrid female turkeys at 28 days of age were exposed to wet litter for 48 h in a randomized block experiment. Expression levels of pro-inflammatory (interleukin (IL)-1 beta, IL-6, and CXCLi2) and signature Th1 (interferon-gamma), Th2 (IL-13) and Treg (IL-10) cytokines were measured in the foot pad tissues using real-time quantitative reverse transcriptase-polymerase chain reaction. Sections of foot pad tissue were stained for CD4+ and CD8+ T lymphocytes, B lymphocytes and macrophages using antibodies that specifically recognize the relevant cell types in the turkey. In the footpads of birds suffering from FPD, there were large fold increases in mRNA expression levels for the pro-inflammatory cytokines IL-1 beta (+635), IL-6 (+65), and CXCLi2 (+1924), and interferon-gamma (+32), whereas there was only a small increase in IL-13 mRNA (+2) and no change in IL-10 mRNA expression levels. CD4+ and CD8+ T lymphocytes were present in the footpads of more than 90% of birds housed on wet litter compared with 25% or less on dry litter. Macrophages were observed in the footpads of approximately 85% of birds housed on wet litter compared with none in birds housed on dry litter. B lymphocytes were not detected in tissue from any of the birds. The data suggest that FPD is associated with a rapidly occurring inflammatory response, rather than a Th2-mediated allergic reaction.

Introduction

Foot pad dermatitis (FPD) is very common in flocks of growing turkeys and is a potential welfare and economic problem in intensive production systems. FPD develops rapidly in commercial flocks, where fully developed lesions occur by 3 weeks of age and from 6 weeks lesions simply increase in size (Mayne et al., 2006, 2007a). FPD is associated with redness, swelling and tissue necrosis, and may also be accompanied by pain (Martland, 1984, 1985; Mayne, 2005; Mayne et al., 2007b). Histopathologically, FPD is associated with massive increases in heterophils and macrophages and the loss of surface keratin (Mayne et al., 2006).

We have developed a simple model for inducing FPD in turkeys that consists of exposing six birds at 28 days of age to clean wet wood shavings in large floor pens for 6 to 8 days (Mayne et al., 2007b). We have also shown that the presence of excreta is not necessary for the development of FPD and that wet litter alone causes similarly severe lesions as wet dirty litter (Mayne et al., 2007b). The rate of progression and extent of lesions are variable both within and between different experiments but external signs of FPD are evident as soon as 24 h after initial exposure. Similar responses to those on wood shavings litter were obtained with paper and cardboard litters, and FPD was worse in birds housed on long barley straw, suggesting that any putative causal factor is not specific to wood shavings (Mayne et al., 2007b). What is not clear is whether FPD in this model represents an inflammatory immune response or an allergic reaction to an environmental stimulus.

Inflammatory responses are driven by pro-inflammatory cytokines and chemokines (typically interleukin (IL)-1 β , IL-6 and IL-8) produced mainly by macrophages. The cytokines cause dilation of local small blood vessels and changes in the endothelial cells lining their walls. This leads to the extravasation of leukocytes, initially heterophils and monocytes, but followed by T lymphocytes and B lymphocytes, into the inflamed tissue, guided by the chemokines produced by the activated macrophages. Plasma proteins and fluids also leak into the tissues as the blood vessels become more permeable. In mammals, dermatitis is typically caused by an inflammatory response, although allergic dermatitis is caused by a Th2 cytokine-driven immunological reaction following previous exposure (sensitization) to an allergen.

Cytokines and chemokines are soluble chemical messengers that regulate all aspects of immune responses. Measurement of the change in levels of specific cytokines can be indicative of the level of inflammation and potential tissue damage. CD4⁺ T lymphocytes are

helper cells (Th) that, through the production of specific cytokine subsets, drive either inflammatory, cell-mediated immune responses (Th1, for which the signature cytokine is interferon (IFN)- γ) or humoral (allergic) immune responses (Th2, for which the signature cytokines are IL-4 and IL-13) or act as regulatory cells (Treg, for which the signature cytokines are IL-10 and TGF- β 1).

We recently characterized the chicken genome's full complement of cytokines and chemokines (Kaiser et al., 2005). The chicken genome encodes orthologues of mammalian IL-1 β , IL-6, IFN- γ , IL-4, IL-13, IL-10 and transforming growth factor (TGF)- β 1 (called TGF- β 4 in the chicken). However, it has a different repertoire of pro-inflammatory chemokines, for which we proposed a simple nomenclature until their biological functions and ligand–receptor relationships are better characterized. For example, in the human IL-8 is a single copy gene. By contrast, the chicken genome contains two genes, CXCLi1 (K60; Sick et al., 2000) and CXCLi2 (IL-8/CAF; Martins-Green & Feugate 1998; Kaiser et al., 1999), both of which have high identity with human IL-8 and share similar biological properties. We have also cloned many of these cytokines and chemokines, and developed reagents to them, to measure their expression in the turkey (Lawson et al., 2001; F. Powell, M. Clarkson & P. Kaiser, unpublished results).

The hypothesis tested in this experiment was that the reaction to wet litter in FPD was caused by an allergy rather than an inflammatory response. Cellular and cytokine responses were evaluated in foot pad tissue from turkeys that were housed on wet or dry wood shavings litter for 48 h. Immunohistochemistry of turkey foot pad sections was carried out to identify CD4⁺ and CD8⁺ T lymphocytes, macrophages and B lymphocytes, and the expression of pro-inflammatory (IL-1 β , IL-6, and CXCLi2), signature Th1 (IFN- γ), Th2 (IL-13) and Treg (IL-10) cytokines was measured using real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods

Birds and husbandry

Seventy-two newly hatched female T8 Large White Broad Breasted turkeys (British United Turkeys, Chester, UK) were obtained from a commercial hatchery. The birds were housed in a single large pen for 7 days and then distributed equally to 12 pens measuring 2 m x 3 m. The pens were littered with clean white wood shavings that had been polythene wrapped by

the suppliers but had not been sterilized. Excreta were removed from all pens daily to maintain clean litter. Each pen contained a hanging bell drinker, a feeder and a suspended heat lamp. The air temperature was maintained at 28°C by controlled ventilation and heating. The daily photoperiod was 14 h of light with 10 h of darkness, and the light intensity was 20 lux throughout the experiment.

Experimental treatments

Starting at 28 days, approximately 10 l tap water was added to the litter of six pens to achieve a litter score of at least 4 on the Tucker and Walker (1999) scale. After 24 h a further 5 l were applied to the wet pens, and excreta and soiled litter were removed. The remaining six pens were kept dry (score 1) and clean by removing excreta and soiled litter.

Observations

At 48 h post-treatment all turkeys were assessed for FPD and assigned an external foot pad score on a seven-point scale (Mayne et al., 2007b). Three birds, two for the experiments and one as a back-up, were randomly selected from each pen and killed with an overdose of sodium pentobarbitone, and the remaining birds were used for another experiment (Mayne et al., 2007b). The dissecting table, instruments and gloves were cleaned with RNAzap (Ambion, Cambridgeshire, UK) and these procedures was repeated between bird dissections. The skin of the foot pad was removed and cut into two pieces. One small piece (approximately 5 mm x 5 mm) was placed on a round cork tile (20 mm x 3 mm), covered in OCT compound (BDH Laboratory Supplies, Dorset, UK), snap-frozen in liquid nitrogen and placed in a zip-lock bag in liquid nitrogen until it could be stored in a -80°C freezer. The second part of the skin of the foot pad was stored in RNAlater (Ambion) in a sterile 15 ml centrifuge tube and kept on ice until being transferred to a refrigerator at 4°C.

Preparation of tissue sections

Sections, 6 µm thick, were cut from the frozen tissue and cork blocks in a Leica CM 1900 cryostat (Leica Microsystems, Wetzlar, Germany) and were mounted on slides. After fixation in ice-cold acetone for 10 min, they were air-dried and then stored at -80°C until use.

Immunohistochemistry

Processed sections were circled with a hydrophobic pen and allowed to dry for 2 min. Foot pad sections were rehydrated with 200 µl phosphate-buffered saline pipetted onto each sample and left for 5 min. Processed sections were stained using a Vectastain ABC α-mouse IgG HRP staining kit (Vector Laboratories, Burlingame, California, USA), following the manufacturer's instructions. Staining for the different cells used the following murine anti-chicken antibodies, previously demonstrated to cross-react with the equivalent turkey cells (Lawson et al., 2001): CD4⁺ (AV29, 1:5 dilution), CD8⁺ (11-39, 1:5 dilution), macrophage (KuL01, 1:500 dilution) and B lymphocytes (AV10, 1:5 dilution). NovaRed (200 µl) was pipetted onto each sample and left for between 30 s and 3 min depending on the colour intensity required (Vector NovaRED substrate kit SK-4800; Vector Laboratories). Slides were allowed to dry overnight before mounting with a cover slip using Surgipath Clearium Mounting Medium (Surgipath, Illinois, USA). Slides stained for specific cell types, and foot pad sections stained with haematoxylin and eosin, were examined under a light microscope and samples with positive staining were recorded.

RNA extraction and real-time quantitative RT-PCR

Approximately 30 mg foot pad tissue was homogenized in 600 µl lysis buffer (RLT; Qiagen, Crawley, UK) using a bead mill (Retsch MM300; Retsch UK Ltd, Leeds, UK). Complete disruption of tissue was ensured by using a QIAshredder (Qiagen) following the manufacturer's instructions. Total RNA was prepared from the homogenized tissues using an RNeasy mini kit (Qiagen), again following the manufacturer's instructions. Purified RNA was eluted in 50 µl RNase-free water and stored at -70°C.

Cytokine and chemokine mRNA levels in foot pad tissues were quantified using a previously described method (Kaiser et al., 2000, 2003; Kogut et al., 2003a, b; Peters et al., 2003; Sijben et al., 2003). Turkey IFN-γ had been cloned previously (Lawson et al., 2001). The remaining turkey cytokine and chemokine cDNAs (except IL-6) were cloned and sequenced (F. Powell & P. Kaiser, unpublished data). For IL-1β, IL-6 and the housekeeping gene 28S, the previously described chicken primer-probe sets could be used. For others (CXCLi2, IL-10 and IL-13), turkey-specific primer-probe sets were developed.

Primers and probes were designed using the Primer Express software program (Applied Biosystems, Foster City, California, USA); details are presented in Table 1. For all cytokines and chemokines, either a primer or probe was designed from the sequence of the relevant

genes to lie across intron–exon boundaries. All probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher *N,N,N,N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

Real-time quantitative RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following cycle profile: one cycle of 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, and 40 cycles of 94°C for 20 sec, 59°C for 1 min. Quantification was based on the increased fluorescence detected due to hydrolysis of the target-specific probes by the 5'-exonuclease activity of the *Tth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-rhodamine, which is not involved in amplification, was used for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye passes a significance threshold (ΔR_n).

To account for variation in sampling and RNA preparation, the C_t values for cytokine-specific or chemokine-specific product for each sample were standardized using the C_t value of the 28S rRNA product for the same sample. To normalize RNA levels between samples within an experiment, the mean C_t value for the 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube to tube variations in 28S rRNA C_t values about the experimental mean were calculated. The slope of the 28S rRNA \log_{10} dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine, chemokine or 28S rRNA \log_{10} dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific and chemokine-specific C_t values as follows: corrected C_t value = $C_t + (N_t - C_t') \times S/S'$, where C_t is the mean sample C_t , N_t is the experimental 28S mean, C_t' is the mean 28S of sample, S is the cytokine or chemokine slope, and S' is the 28S slope. Results were expressed as $40 - C_t$ values and each sample was assayed in triplicate.

Statistical analysis

The experiment was a randomized block design with two blocks of six pens and two treatments (wet and dry litter). External foot pad scores were analysed with a one-way analysis of variance of pen means ($n=3$). Cellular responses were compared by maximum

likelihood chi-squared test of the number of birds with or without positive staining in the two treatments ($n=12$). Results for quantitative RT-PCR were analysed using a split plot analysis of variance of pen means ($n=2$).

Results

The mean external scores of FPD of turkeys after 48 h on wet and dry litter, respectively, were 6.2 and 1.2 on a seven-point scale (Mayne *et al.*, 2007b), illustrating the extent of inflammation that can be caused in a comparatively short period of time by housing birds on wet litter. Furthermore all the birds on the wet litter treatment were strongly affected: the range of external foot pad scores was 5 to 7, compared with 0 to 3 on the dry treatment.

Cell staining

Typical sections stained for macrophages, for CD4⁺ and CD8⁺ T lymphocytes, and for B lymphocytes respectively for birds on wet and dry litter with different external footpad scores are presented in Figures 1–4. Positively stained cells are dark red–brown. Artefacts of processing, likely to be contaminants, were present in most sections and appeared as large black particles, greater in size and darker in colour than positively stained cells. Artefacts of processing also had an uneven edge, while cells were more regularly shaped. The pale brown background stain on all sections was non-specific.

Positive staining for macrophages (Figure 1) was seen in 85% of birds (10 of 12) housed on wet litter within the uppermost layer of the epidermis just below the keratin layer (surface keratin had been lost), whereas there was no staining for macrophages in birds housed on dry litter ($\chi^2=21.8$, $P<0.001$). CD4⁺ T lymphocytes (Figure 2) were evident throughout the upper and lower dermis of all birds housed on wet litter compared with only 15% (two of 12) housed on dry litter ($\chi^2=15.5$, $P<0.001$). Positive staining for CD8⁺ T lymphocytes (Figure 3) was present in over 90% of birds (11 of 12) housed on wet litter compared with 25% of birds (three of 12) housed on dry litter ($\chi^2=13.7$, $P<0.001$). No staining for B lymphocytes was observed in any of the sections (Figure 4).

Quantitative RT-PCR

Means, standard errors and fold changes of corrected $40 - C_t$ values for each cytokine and chemokine are presented in Table 2. There was a significant difference ($P<0.05$) between the

mRNA expression levels of the majority of cytokines and chemokines measured in the foot pad tissues of birds housed on wet and dry litter after 48 h; the only exception being the Treg cytokine IL-10, for which there was no significant difference in mRNA expression levels. For the pro-inflammatory cytokines, mRNA expression levels were upregulated dramatically in foot pad tissues from birds housed on wet litter, as opposed to those housed on dry litter, from 65-fold for IL-6 to 1924-fold for CXCLi2. IFN- γ mRNA expression levels were also greatly increased (32-fold). IL-13 mRNA expression levels, although significantly different, were only increased two-fold.

Discussion

Our primary objective was to determine whether the inflammation associated with FPD in turkeys was due to an allergic reaction to an allergen in the litter, or to a classical inflammatory response. To this end, we measured cytokine mRNA expression levels, and used immunohistochemistry to identify subsets of immune cells, in the footpad tissues in a previously validated model for inducing FPD in turkeys (Mayne *et al.*, 2007b).

In previous experiments a marked inflammatory response was observed by 48 h after exposure to wet litter, and we chose this time point to ensure that a severe immune response was observed and also to avoid any secondary reparative changes. Footpad tissue sections stained for specific immune cell subsets showed that birds housed on wet litter had increased levels of macrophages and CD4⁺ and CD8⁺ T lymphocytes, but no detectable B cells. Also, mRNA expression levels for the pro-inflammatory cytokines IL-1 β , IL-6 and CXCLi2 were all highly upregulated in these tissues, with CXCLi2 increasing by more than 1900-fold. Pro-inflammatory reactions commonly lead to a Th1, IFN- γ -mediated response that results in an influx of CD8⁺ cytotoxic T cells, whereas only 25% of birds housed on dry litter showed positive staining for CD8⁺ T lymphocytes. This was not unexpected, however, as naïve T lymphocytes only respond in substantial numbers to an inflammatory reaction 4 to 5 days post initial inflammation. For the Th signature cytokines, both IFN- γ and IL-13 mRNA expression levels were upregulated, the former by more than 30-fold, whereas IL-13 only increased two-fold, a difference that would not normally be considered of biological significance.

In contrast to our results for macrophage staining, Igyarto *et al.* (2006) reported that 50% of epidermal dendritic cells were positive for the KuL01 antibody in featherless skin of 8-week-

old chickens. We failed to detect macrophage cells in the footpad of birds on the dry treatment, and the differences between the two data sets may be a consequence of different housing, age, tissue or species and warrant further research.

The results are consistent with the conclusion that FPD in these turkeys was a non-specific inflammatory reaction. Although an allergic reaction, perhaps to a water-soluble antigen in the litter released when the litter was wet, could have occurred, it is unlikely that this would have happened within 48 h unless the turkeys had been previously exposed to the allergen causing the reaction. Damp patches are inevitably present in normal dry litter and it is probable that the skin of the foot pads of these turkeys was exposed to water-soluble allergens in the period before the experiment. If sensitization to these allergens had occurred, exposure to the experimental treatment should induce a secondary allergic response in 48 hours, which we did not observe: the cytokines that would be indicative of an allergic response (IL-10 and IL-13) were not recorded at high levels.

In conclusion, these data suggest that there was a rapidly occurring inflammatory response in the foot pads of birds housed on wet litter for 48 h and that an allergic response was unlikely. Further research is required to clarify the mode of action of litter moisture in the aetiology of turkey FPD.

Acknowledgments

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Legends

Tab.1

Real-time quantitative RT-PCR probes and primers

Tab.2

Corrected cytokine $40 - C_t$ values for foot pad tissue of turkeys raised on dry litter and exposed to dry or wet litter for 48 h at 28 days of age.

Fig.1

Presence of macrophages in footpad tissue sections from turkeys housed on (1a) dry or (1b, 1c) wet litter for 48 h. Original magnification: x10 (1a, 1b) or x20 (1c). External footpad scores were 0 (1a) and 7 (1b, 1c). A, keratin; B, lost surface keratin; C, epidermis; D, dermis; E, positively stained cells in epidermis and dermis. Bar: 100 mm (1a, 1b) or 50 mm (1c).

Fig.2

Presence of $CD4^+$ T lymphocytes in footpad tissue sections from turkeys housed on (2a) dry or (2b, 2c) wet litter for 48 h. Original magnification: x10 (2a, 2b) or x20 (2c). External footpad scores were 0 (2a) and 7 (2b, 2c). A, keratin; C, epidermis; D, dermis; E, positively stained cells in epidermis and dermis. Bar: 100 mm (2a, 2b) or 50 mm (2c).

Fig.3

Presence of $CD8^+$ T lymphocytes in footpad tissue sections from turkeys housed on (3a) dry or (3b, 3c) wet litter for 48 h. Original magnification: x10 (3a, 3b) or x20 (3c). External footpad scores were 3 (3a) and 6 (3b, 3c). A, keratin; B, lost surface keratin; C, epidermis; D, dermis; E, positively stained cells in epidermis and dermis. Bar: 100 mm (3a, 3b) or 50mm (3c).

Fig.4

Absence of B lymphocytes in footpad tissue sections from turkeys housed on (4a) dry or (4b) wet litter for 48 h. Original magnification: x10. External footpad scores were 0 (4a) and 7 (4b). A, keratin; C, epidermis; D, dermis. Bar: 100 mm (4a, 4b).

Fig.1

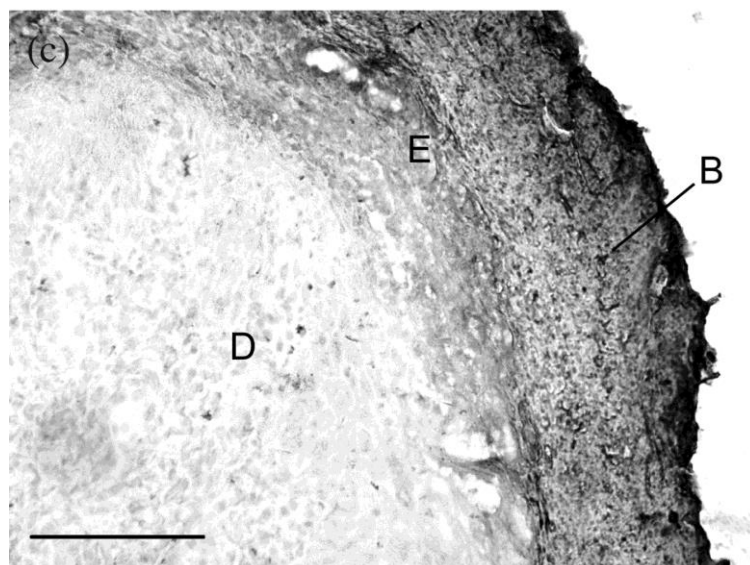
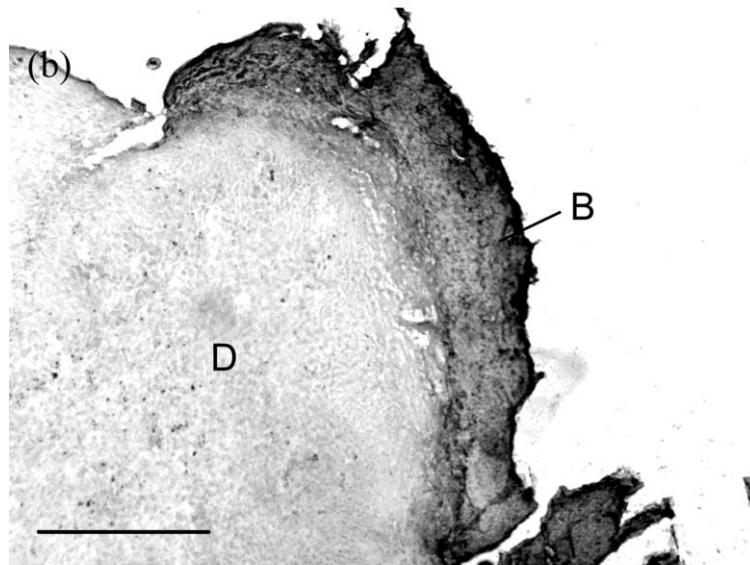
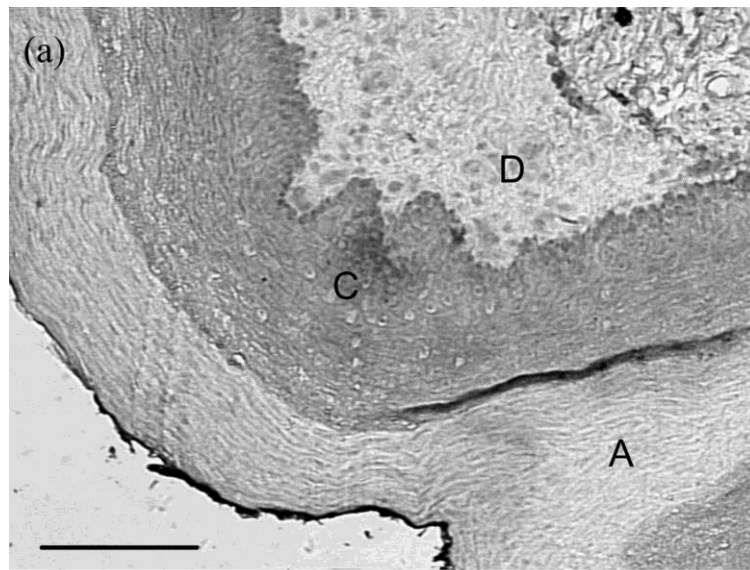


Fig.2

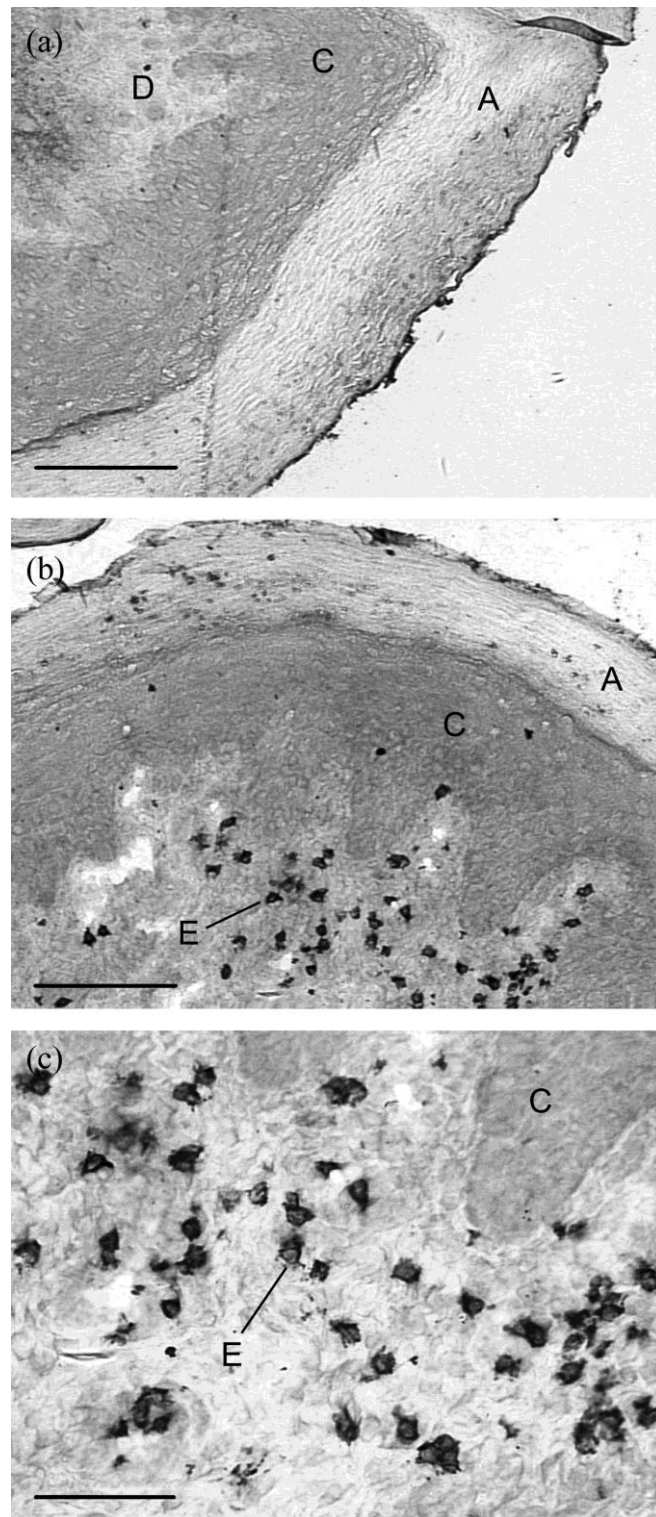


Fig.3

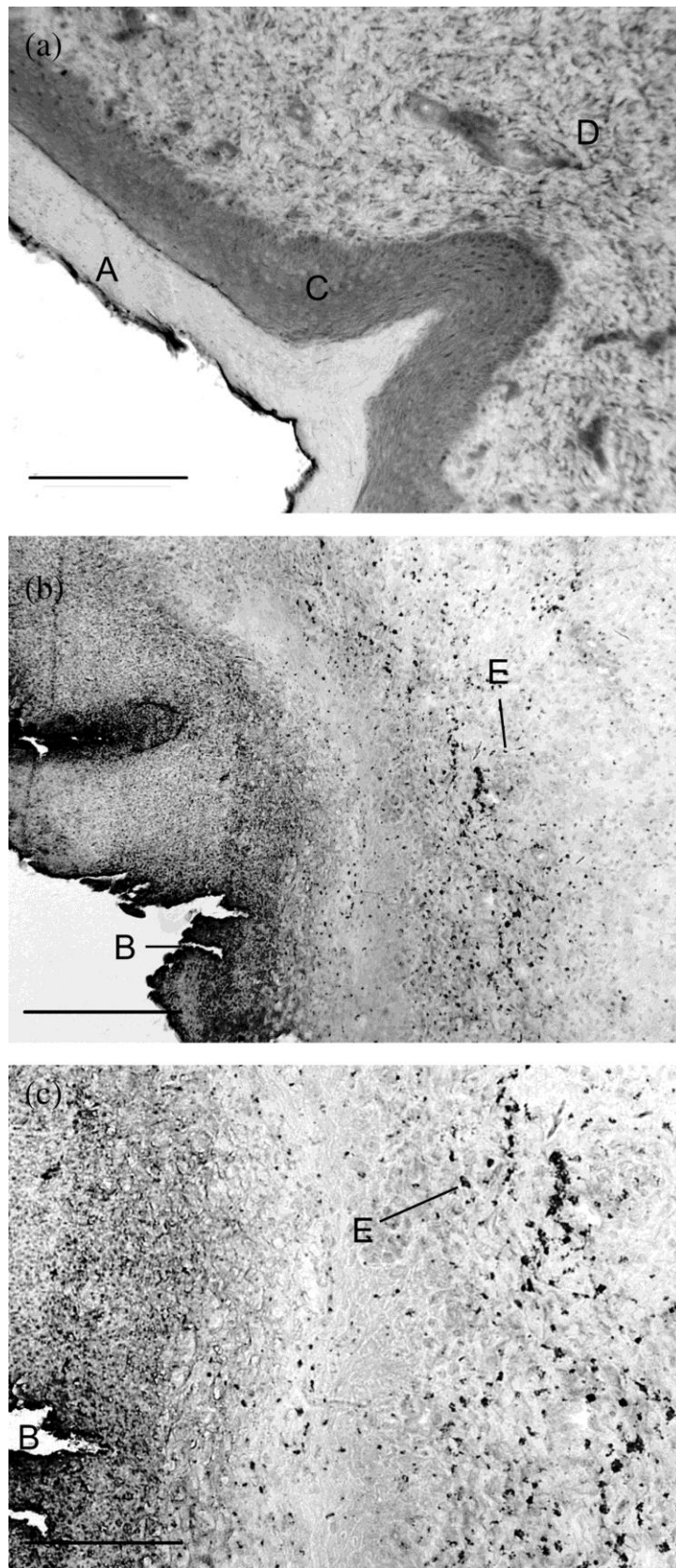
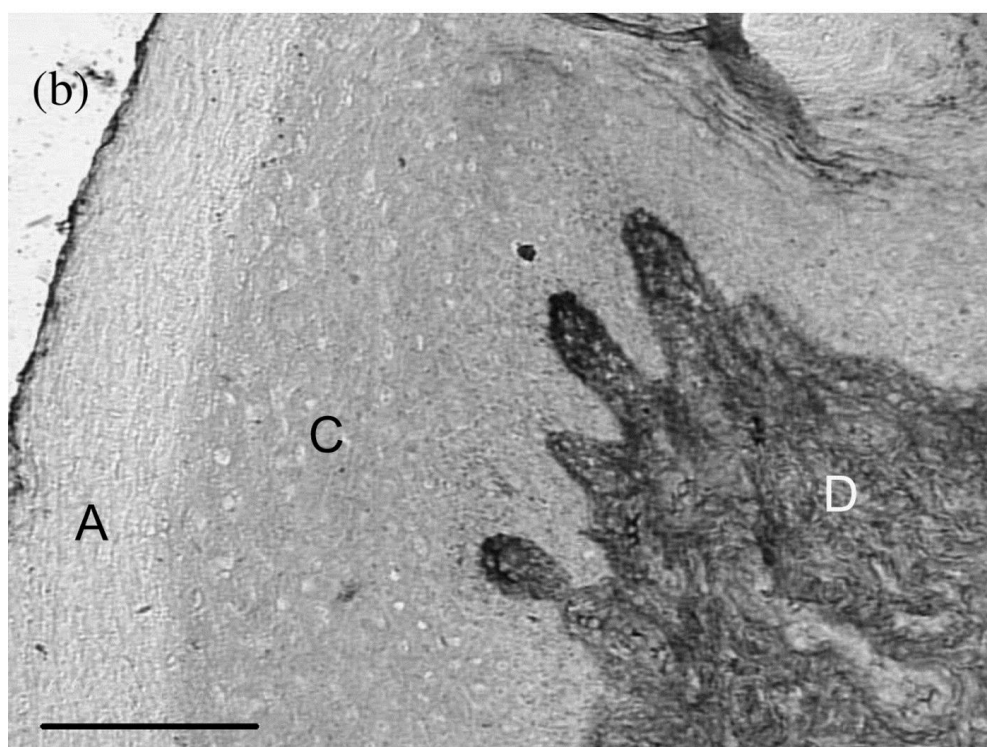
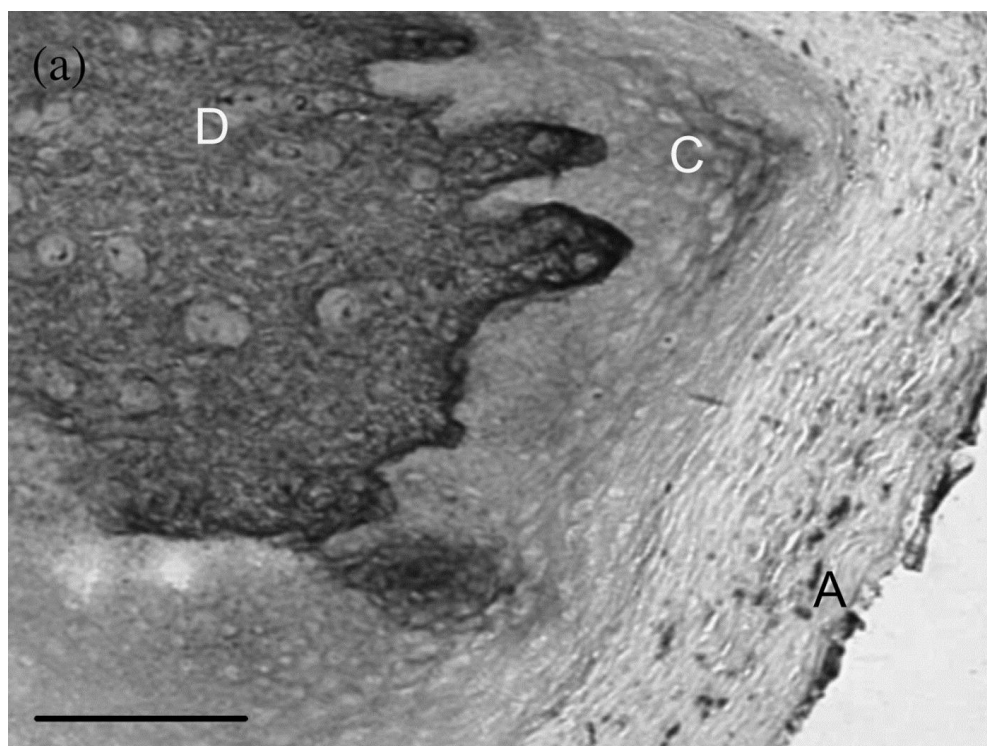


Fig.4



Tab.1

RNA target	Probe/primer sequence	Specific for	Accession number	Exon boundaries ^a
28S		Chicken and turkey	X59733	—
Probe	5'-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3'			
Forward primer	5'-GGCGAAGCCAGAGGAAACT-3'			
Reverse primer	5'-GACGACCGATTTGCACGTC-3'			
IL-1 β		Chicken and turkey	AJ245728	5/6
Probe	5'-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3'			
Forward primer	5'-GCTCTACATGTCGTGTGTGATGAG-3'			
Reverse primer	5'-TGTCGATGTCCCGCATGA-3'			
IL-6		Chicken and turkey	AJ250838	3/4
Probe	5'-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3'			
Forward primer	5'-GCTCGCCGGCTTCGA-3'			
Reverse primer	5'-GGTAGGTCTGAAAGGCGAACAG-3'			
CXCLi2		Turkey	AM493430	1/2

RNA target	Probe/primer sequence	Specific for	Accession number	Exon boundaries^a
Probe	5'-(FAM)-CAGCTCTGTCACAAGGTAGGACCCTGGT-(TAMRA)-3'			
Forward primer	5'-TGGCTCTCCTCCTGGTTTCA-3'			
Reverse primer	5'-GCAGCTCGTTCCCCATCTT-3'			
IFN- γ		Turkey	AJ000725	3/4
Probe	5'-(FAM)-AAAGATATCATGGACCTGGCCAAGCTTCA-(TAMRA)-3'			
Forward primer	5'-AACCTTCCTGATGGCGTGAA-3'			
Reverse primer	5'-CTTGCGCTGGATTCTCAAGTC-3'			
IL-13		Turkey	AM493431	3/4
Probe	5'-(FAM)-TGCCAGCTGAGCACCGACAACG-(TAMRA)-3'			
Forward primer	5'-CCTGCACGGCCAGATGA-3'			
Reverse primer	5'-GGCAAGAAGTTCCGCAGGTA-3'			
IL-10		Turkey	AM493432	3/4
Probe	5'-(FAM)-CCTGAAGATGACAATGAAGCGCTGTCA-(TAMRA)-3'			
Forward primer	5'-CGACCTGGGCAACATGCT-3'			
Reverse	5'-CCTCTCGCAGGTGAAGAAGTG-3'			

RNA target	Probe/primer sequence	Specific for	Accession number	Exon boundaries^a
primer				
^a Exon boundaries in the chicken gene (gene structures have not yet been determined for the turkey genes).				

		Corrected 40 – C_t value				
Cytokine/chemokine		Dry litter	Wet litter	SED ^a	<i>P</i>	Fold change ^b
Pro-inflammatory	IL-1 β	3.66	12.97	0.854	<0.001	+635
	IL-6	7.50	10.51	1.057	0.019	+65
	CXCLi2	10.10	21.01	1.014	<0.001	+1924
Th1	IFN- γ	1.44	6.45	0.563	<0.001	+32
Th2	IL-13	7.43	8.52	0.262	0.002	+2
Treg	IL-10	4.02	2.51	0.780	0.086	–
^a SED: Standard error of difference between treatment measurements. ^b Calculated as 2[(wet litter 40 – C_t value) – (dry litter 40 – C_t value)].						